A | #08/484,594 O'Brien et al.
Filed: 06/07/95 MYELOS.002DV2
Exhibit D

Vol. 204, No. 2, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 28, 1994 Pages 994-1000

PROTECTION BY PROSAPOSIN AGAINST ISCHEMIA-INDUCED LEARNING DISABILITY AND NEURONAL LOSS

Akira Sano[1§], Seiji Matsuda[2], Tong-Chun Wen[2], Yasunori Kotani[1], Keiji Kondoh[1], Shu-ichi Ueno[1], Yasuo Kakimoto[1], Hiroyuki Yoshimura[3] and Masahiro Sakanaka[2]

Departments of [1]Neuropsychiatry and [2]Anatomy, and [3]Central Research Laboratory, Ehime University School of Medicine, Shigenobu, Onsen-gun, Ehime 791-02, Japan

Received August 27, 1994

Prosaposin, the protein precursor of saposins A, B, C, and D which activate sphingolipid hydrolases, is abundant in several brain regions including the hippocampus. We infused prosaposin continuously for 7 days into the lateral ventricle of gerbils starting 3 hours before 3-min of forebrain ischemia. Using the step-down passive avoidance task, we demonstrated that ischemia-induced learning disability is prevented almost completely by prosaposin infusion. Subsequent light and electron microscopic examinations showed that pyramidal neurons in the CA1 field of the hippocampus as well as synapses within the strata moleculare, lacunosum / radiatum and oriens of the field were significantly more numerous in gerbils infused with prosaposin infusion than in those receiving saline infusion. These findings suggest that prosaposin possesses neurotrotrophic activity to protect hippocampal CA1 neurons from lethal ischemic damage. © 1994 Academic Press, Inc.

Saposins are small glycoproteins that activate hydrolysis of sphingolipids by specific hydrolases (1, 2). The cDNA analysis of saposins has demonstrated that there is a precursor protein, named prosaposin, which contains four saposin domains (3, 4, 5). The amount and processing pattern of prosaposin varies from tissue to tissue. Processed saposins have been shown mainly in the spleen, liver and kidney, whereas the precursor form (prosaposin) appears to be produced chiefly in brain and muscle (6). Immunohistochemical studies show that prosaposin is located exclusively in certain neurons and nerve fibers within the brain (7). Prosaposin can already be detected in the rat brain at birth and gradually increases in content after postnatal day 10 when synaptogenesis begins to take place in the brain (6). Collard et al. reported that prosaposin is released into seminiferous tubules of the testis at specific stages of spermatogenesis (8). An in vitro study has shown that prosaposin can bind to several gangliosides, including GM1 ganglioside (9), which has been shown to protect neurons from damage (10). These findings support the hypothesis that prosaposin participates in neuroprotection or neurotrophism, although in vivo experimental evidence for this speculation has not been demonstrated.

§To whom correspondence should be addressed. Fax: 81-899-64-2336.

0006-291X/94 $5.00
Copyright © 1994 by Academic Press, Inc.
All rights of reproduction in any form reserved.

BEST AVAILABLE COPY

Prosaposin-containing neural elements are abundant in field CA1 of the hippocampus (7) which is known to be vulnerable to ischemic insult. To investigate the in vivo function of prosaposin, we studied its protective effect on ischemia-induced learning disability and neuronal loss in gerbils.

MATERIALS AND METHODS

Animals

Male Mongolian gerbils weighing 70-80 g (about 12 weeks of age) were housed communally at constant temperature (22 ±1°C) with a 12:12 h light-dark cycle, and given food and water ad libitum. They were handled once a week for cage cleaning. The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine.

Osmotic minipump implantation

The animals were anesthetized with 1.5 % halothane in a mixture of nitrous oxide and oxygen (1:0.75). An osmotic minipump (Alza Corp., Palo Alto, CA, USA) was implanted subcutaneously into the back of each animal, and a needle from the minipump was placed in the left lateral ventricle.

Prosaposin infusion

Prosaposin was purified from human milk using an affinity chromatography with monospecic antibody as previously described (11). The protein staining of the purified preparation on SDS-PAGE and the Western blotting showed a single band. The sequence of the initial 10 amino acids from N-terminus of the purified protein was identical to the sequence of prosaposin deduced from cDNA. Prosaposin dissolved in phosphate-buffered saline (PBS) in a dose of 100 or 240 ng/day was infused for 7 days into the lateral ventricles of gerbils in which 3-min forebrain ischemia had been induced; control animals received saline infusion or infusion of PBS with 0.5% bovine serum albumin (BSA). The infusion was started 3 h before the ischemic insult.

Occlusion of the common carotid arteries

The gerbils were anesthetized as described above and fixed to a stereotaxic apparatus. Both common carotid arteries were exposed through a ventral midline incision and separated carefully from the adjacent veins and nerves. Immediately after the cessation of inhalation anesthesia, the common carotid arteries were clamped for 3 minutes with aneurysm clips. Brain and rectal temperatures were maintained at 37±0.2°C during forebrain ischemia. The rectal temperature was monitored by a thermocouple probe (2455, Yokogawa Electric Co. Ltd. Tokyo), and the brain temperature by another thermocouple probe (TN-800, Unique Med. Corp. Tokyo) inserted into the cerebral cortex.

Passive avoidance task

The gerbils were trained in a conventional step-down passive avoidance apparatus which was divided into a safe platform and an experimental chamber. The experimental chamber (22.5 X 20.0 X 19.5 cm) was made of acrylic fiber with a floor constructed of stainless-steel grids. A scrambled DC constant current shock generator (Muromachi Kikai Co. Ltd. Tokyo) delivered a 0.4 mA scrambled shock through the grid. The safe part (20.0 X 9.5 X 3.0 cm) was also made of acrylic fiber and was fixed to one side of the chamber. Training of passive avoidance was carried out at 7 days after forebrain ischemia. Each animal was placed initially on the safe platform. When the gerbil stepped down onto the grid floor, it received a foot shock. Although the gerbil went repeatedly up and down between the platform and the grid, it eventually remained on the platform. This training session lasted for 300 sec. Twenty-four hours later, the gerbil was again placed on the safe platform while the shock generator was turned off, and the response latency, i.e. the time till it stepped down to the grid floor, was measured. This test session also lasted 300 sec.

Histopathological study of hippocampal CA1 region

After passive avoidance experiments, the animals were injected with bromophenol blue through the needles of the minipumps to show dye diffusion into the cerebral ventricles. Then they were anesthetized with pentobarbital and perfused transcardially with 4 % paraformaldehyde -1 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for light and electron microscopy. After perfusion the dorsal hippocampus was removed and three sections 100 μm thick transverse to the axis of the dorsal hippocampus were cut with a microslicer for electron microscopy, and the remaining dorsal hippocampus was embedded in paraffin. Coronal sections were cut from the paraffin-embedded brain and stained with 0.1% cresyl violet. Viable neurons in the hippocampal CA1 field were counted with an image analyzer (Nexus Co. Ltd, Tokyo) and recorded as neuronal density per 1 mm linear length.

BEST AVAILABLE COPY

For electron microscopy, the sections were embedded in epoxy resin. Ultrathin sections containing all strata of field CA1 were cut with a Reichert ultramicrotome and mounted on single-slot (2 X 0.5 mm) grids which were coated with Formvar film. Electron micrographs were taken at the central area of each stratum (15 μm X 18.75 μm = 280 μm^2), and intact synapses in the area were counted.

Statistics

The effect of prosaposin was evaluated by the Mann-Whitney U-test which enabled us to compare the prosaposin-treated group with the vehicle control group after the Kruskal-Wallis one-way analysis of variance.

RESULTS

Prosaposin infusion into the lateral ventricle starting 3 hours before forebrain ischemia and continuing for 7 days, caused a significant dose-dependent prolongation in response latency in the step-down passive avoidance task, when compared with the saline- and PBS/BSA-infusion groups (Fig. 1). The mean of the response latency in ischemic gerbils with 240 ng/day of prosaposin infusion was similar to that of sham-operated animals with saline infusion, suggesting that prosaposin prevented almost completely the occurrence of ischemia-induced learning disability. Subsequent histological examinations revealed that prosaposin treatment rescued many ischemic CA1 neurons which were destined to degenerate without the treatment (Fig. 2A). This finding was confirmed by neuron-counting in the CA1 field of the hippocampus (240 ng/day prosaposin treatment, 203 ± 11 cells/mm, v.s., saline infusion, 137 ± 12, $p < 0.01$) (Fig. 2B). The mean number of viable CA1 neurons in the gerbils treated with the higher dose of prosaposin was, if not the same as, close to that in the sham-operated animals.

In line with the results of the passive avoidance task and light microscopic observations, synapses within the stratum moleculare, stratum lacunosum / radiatum and the stratum oriens of the

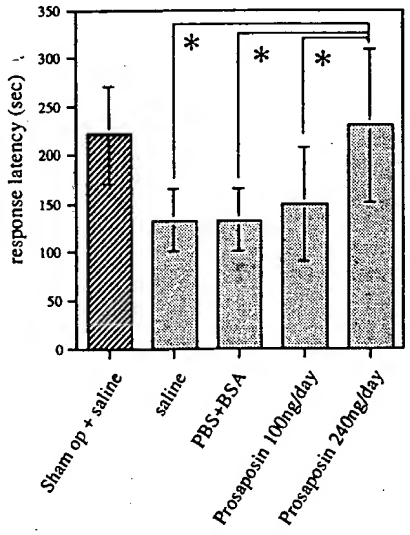
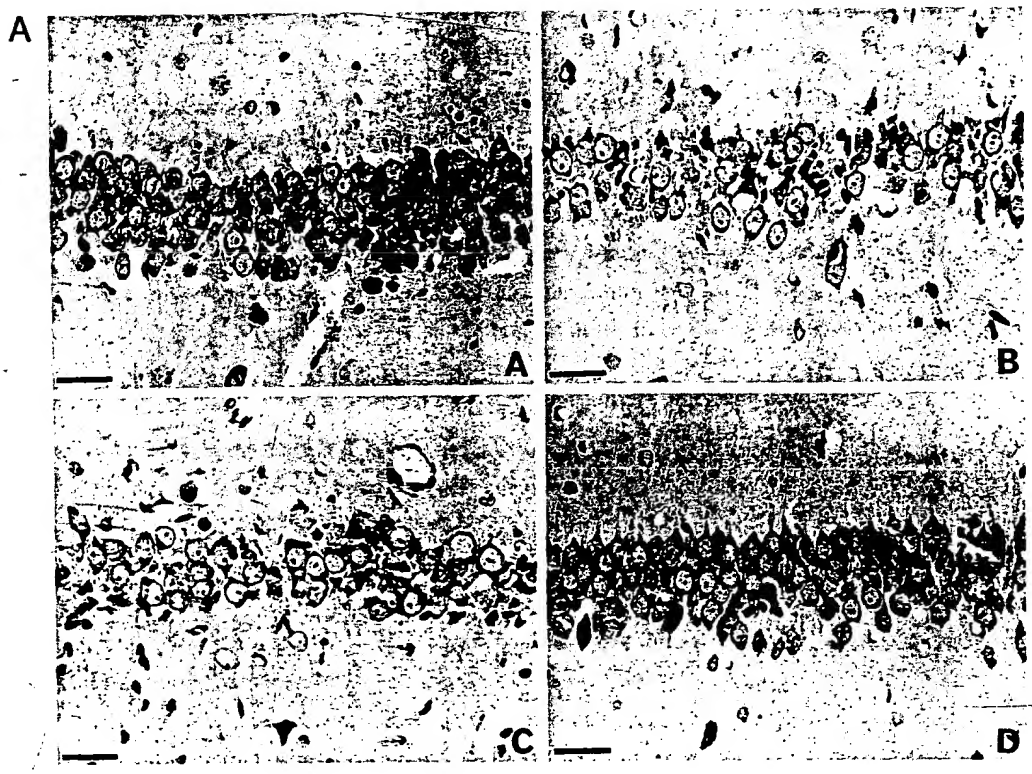


Fig. 1. Preventive effect of prosaposin on learning disability in 3-min ischemic gerbils. Treatment with prosaposin (240 ng/day) abolished almost completely ischemia-induced learning disability as revealed by the passive avoidance task. Each value represents mean ± S.E. (n=6-8). * $P < 0.05$, significantly different from the corresponding saline or PBS/BSA infusion group.

BEST AVAILABLE COPY



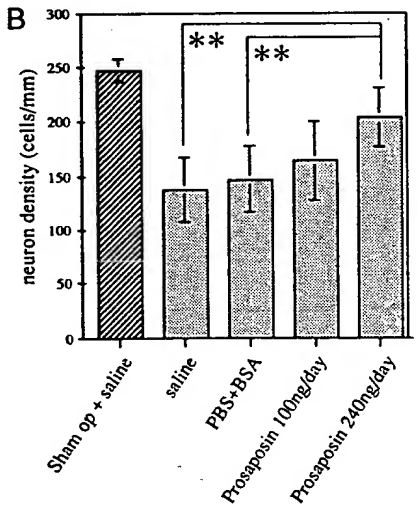


Fig. 2. (A) Photomicrographs of the hippocampal CA1 region of gerbils with or without 3-min ischemia. A: sham-operated animal with saline infusion. B: ischemic animal with saline infusion. C: ischemic animal with prosaposin (100 ng/day) infusion. D: ischemic animal with prosaposin (240 ng/day) infusion. Note that a significant number of hippocampal CA1 pyramidal cells are rescued by the treatment with prosaposin. All sections are stained with cresyl violet. Bar= 100 μm. (B) Effect of prosaposin on the number of hippocampal CA1 neurons. Neurons in the hippocampal CA1 region of ischemic gerbils with saline or PBS/BSA infusion were less numerous than those of sham-operated animals, and the decrease in neuron number in the ischemic hippocampus was prevented significantly in a dose-dependent manner when prosaposin was infused into the lateral ventricle for 7 days. Each value represents mean ± S.E. (n=6-8). ** $P < 0.01$, significantly different from the corresponding saline or PBS/BSA infusion group.

BEST AVAILABLE COPY

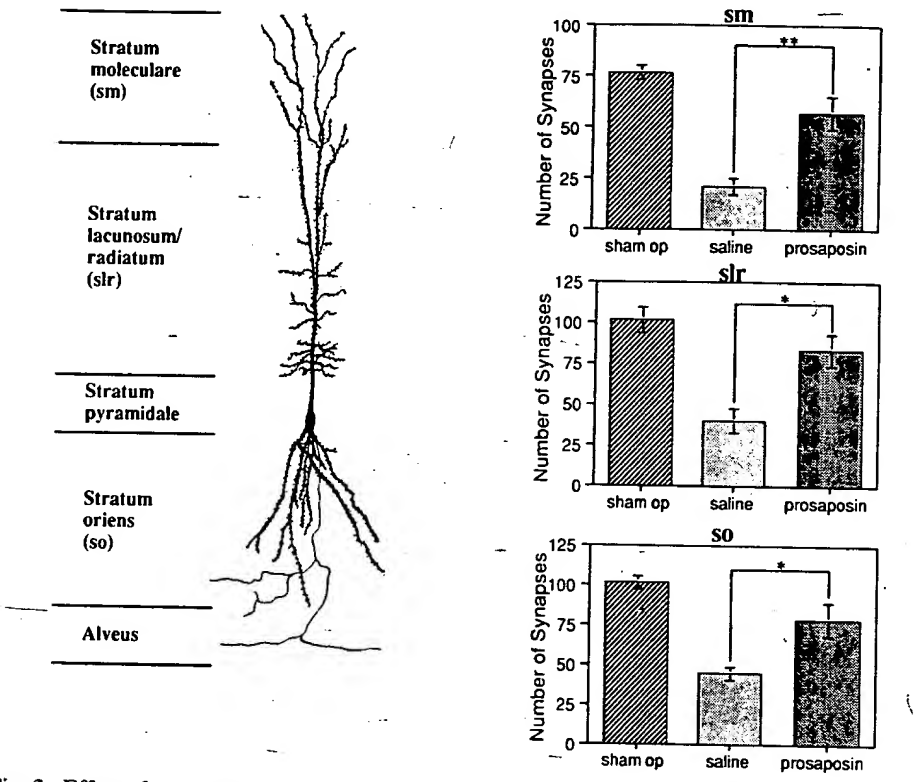


Fig. 3. Effect of prosaposin on the number of synapses in three strata of the ischemic hippocampal CA1 region. Synapses in the strata moleculare, lacunosum / radiatum and oriens of the hippocampal CA1 region of ischemic gerbils with saline or PBS/BSA infusion were less numerous than those in the tree strata of sham-operated animals, and the decrease in synapse number in the ischemic hippocampus was prevented significantly when prosaposin in a dose of 240 ng/day was infused into the lateral ventricle for 7 days. Each value represents mean ± S.E. (n=7-8). * $P < 0.05$, ** $P < 0.01$, significantly different from the corresponding saline infusion group. Redrawn from S. Ramón y Cajal, Histologie du systéme nerveux de l'homme et des vertebris. Tome II, Maloine. Paris (1911).

hippocampal CA1 region, as revealed by electron microscopy, were more numerous in prosaposin-treated than in non-treated ischemic gerbils (Fig. 3).

DISCUSSION

During forebrain ischemia, brain temperature has been shown to fall differently in individual animals, thereby affecting the number of viable CA1 neurons after ischemia (12). To avoid the effect of unstable brain temperature on ischemic neuronal loss, we kept the brain temperature at 37.0 ± 0.2°C while clamping the common carotid arteries. This enabled us to apply the same intensity of ischemic insult to all animals and to evaluate with accuracy the neuroprotective effect of prosaposin on 3-min ischemic gerbils which exhibit degeneration of nearly half the neurons in the hippocampal CA1 region unless neuroprotective agents are administered. Taken together, the present study provides a simple and useful tool for functional and morphological analyses of putative neuroprotective molecules.

BEST AVAILABLE COPY

It is not always possible to compare the neuroprotective activity of prosaposin with that of other growth factors so far examined; however, prosaposin is more potent than basic fibroblast growth factor with respect to the trophism of ischemic neurons (13).

A human patient and his fetal sibling with total deficiency of prosaposin, which was caused by a mutation in the initiation codon was reported (14, 15). Shortly after birth the patient had severe neurological deficits, and died with serial generalized convulsive attacks at the age of 16 weeks. Although autopsy was not performed, a computed tomography of the brain at the age of 3 weeks showed multicentric hypodense areas. Biochemical studies of the 20 week-old fetal sibling did not show increase in any sphingolipids in the brain, but the liver and kidney of the fetus exhibited multi-substrate sphingolipidosis. These findings suggest that brain prosaposin is involved in a central event different from the generation of saposins and that it is indispensable for normal development of the central nervous system.

Prosaposin can bind in vitro to several gangliosides including GM1-ganglioside and GQ1b-ganglioside, which are rich in neuronal cell membrane (9). Many gangliosides are known to modify neuronal functions and GM1-ganglioside has been shown to exert protective effects on damaged neurons (10). Prosaposin may participate in neurotrophism through its binding to and interaction with several gangliosides on cell surface membrane after being secreted from nerve endings into synaptic clefts or extracellular spaces.

In conclusion, the present study demonstrated that prosaposin can protect hippocampal CA1 neurons from lethal ischemic damage, and it opens a new research field of prosaposin as a neurotrophic factor candidate.

ACKNOWLEDGMENTS

This work was supported, in part by a grant from Uehara Memorial Foundation (AS, MS). The authors are grateful to Ms. Kazumi Matsumoto for her technical assistance. Dr. Tong-Chun Wen is on leave of absence from Taishan Medical College, Shandong Province, People's Republic of China.

REFERENCES

1. O'Brien J. S. and Kishimoto Y. (1991) FASEB J. 5, 301-308
2. Fürst W. and Sandhoff K. (1992) Biochim. Biophys. Acta 1126, 1-16
3. O'Brien J. S., Kretz K. A., Dewji N., Wenger D. A., Esch F., and Fluharty A. L. (1988) Science 241, 1098-1101
4. Roman E. G. and Grabowski G. A. (1989) Genomics 5, 486-492
5. Nakano T., Sandhoff K., Stümper J., Christomanou H., and Suzuki K. (1989) J. Biochem. (Tokyo) 105, 152-154
6. Sano A., Hineno T., Mizuno T., Kondoh K., Ueno S., Kakimoto Y., and Inui K (1989) Biochem. Biophys. Res. Comm. 165, 1191-1197
7. Kondoh K., Sano A., Kakimoto Y., Matsuda S., and Sakanaka M. (1993) J. Comp. Neurol. 334, 590-602
8. Collard M. W., Sylvester S. R., Tsuruta J. K., and Griswold M. D., (1988) Biochemistry 27, 4557-4564
9. Hiraiwa M., Soeda S., Kishimoto Y., and O'Brien J. S. (1992) Proc. Natl. Sci. Acad. USA 89, 11254-11258
10. Geisler F. H., Dorsey F. C.,and Coleman W. P. (1991) N. Engl. J. Med. 324, 1829-1838

BEST AVAILABLE COPY

11. Kondoh K., Hineno T., Sano A., and Kakimoto Y. (1991) Biochem. Biophys. Res. Comm. 181, 286-292
12. Mitani A., Andou Y., Matsuda S., and Kataoka K. (1991) Neurosci. Lett. 131, 171-174
13. Nakata N., Kato H., and Kogure K. (1993) Brain Res. 605, 354-356
14. Harzer K., Paton B. C., Poulos A., Kustermann-Kuhn B., Roggendorf W., Grisar T., and Popp M. (1989) Eur. J. Pediat. 149, 31-39
15. Schnabel D., Schröder M., Fürst W., Klein A., Hurwitz R., Zenk T., Weber J., Harzer K., Paton B. C., Poulos A., Suzuki K., and Sandhoff K. (1992) J. Biol. Chem. 267, 3312-3315

BEST AVAILABLE COPY

In the article "Rele
by Shih-fang Fan, Shuya V
On page 28, line
protein kinase c can be
activated and...."

In the article "A
Pancreatic Islets," by Qi
Okamoto, Pamela J. Kai
Seino, pages 629-636:
On page 634, Fi
correct as printed.